the two inhibitors is manifested completely in the association rate constants; for **6** k_{on} is 6600 s^{-1} M^{-1}, and for **7** k_{on} is 470000 s^{-1} M^{-1}. Interestingly, Laskowski (personal communication) has made a similar observation in the case of the natural protein protease inhibitors: the change of arginine for the valine manifests most of its effect in the association rate constant. The k_{off} values are very similar and can essentially be considered identical, since there is a large amount of error in this parameter when determined by the Cha method.

In the design of these small synthetic inhibitors of α-chymotrypsin we have attempted (successfully) to mimic the important interactions that have been observed in both good substrates and effective natural protease inhibitors. With this general strategy in mind, and with the viable difluoromethyl ketone functional group at the center of the inhibitor, it should be possible, by observing the sequence of both natural inhibitors and good substrates, to design effective small (M_r <1000) synthetic inhibitors to any of the physiologically important serine proteases.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Detailed description of the synthesis, purification, and physical properties of all new compounds used in this study (14 pages). Ordering information is given on any current masthead page.

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Adduct Formation between the Cupric Site of Phenylalanine Hydroxylase from *Chromobacterium violaceum* and 6,7-Dimethyltetrahydropterin†

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ABSTRACT: The interaction of pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* with the cofactor analogue 5-deaza-6-methyltetrahydropterin and the cofactor 6,7-dimethyltetrahydropterin ($DMPH_4$) has been investigated by multifrequency electron spin resonance (ESR) spectroscopy. 5-Deaza-6-methyltetrahydropterin, which lacks the N-5 nitrogen present in the pyrazine ring of $DMPH_4$, binds tightly to the cupric form of the enzyme; however, no changes are observed in the ESR parameters of the copper center. In contrast, the binding of $DMPH_4$ (or 6-methyltetrahydropterin) shifts the ESR parameters ($g_{\|}$ and $A_{\|}$) associated with the cupric enzyme. In addition, superhyperfine transitions were resolved and assigned to hyperfine splitting from nitrogen ligands. ESR spectra of the enzyme recorded in the presence of [5-^{14}N]$DMPH_4$ or [5-^{15}N]$DMPH_4$ were computer simulated and found to be consistent with pterin serving as a direct donor ligand to the copper center through the N-5 position.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the formation of tyrosine from phenylalanine in the presence of a reduced pterin cofactor and molecular oxygen. In addition, phenylalanine hydroxylase (PAH)[1] from either bacterial or mammalian liver requires a transition metal for activity. While the mammalian liver enzyme contains 1 mol equiv of tightly bound iron (Fisher et al., 1972; Gottschall et al., 1982), the enzyme from *Chromobacterium violaceum* contains 1 mol equiv of copper

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[1] Abbreviations: PAH, phenylalanine hydroxylase; pterin, generic name for 2-aminopteridin-4-one; $DMPH_4$, 6,7-dimethyltetrahydropterin; EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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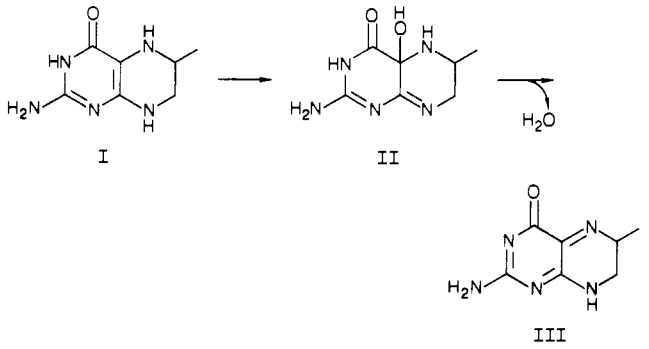
Scheme I



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(Pember et al., 1987). In each case the enzyme must be reduced to the ferrous or cuprous form for catalytic activity (Wallick et al., 1984; Pember et al., 1987). A 4a-hydrate of the pterin cofactor is formed concomitantly with hydroxylation during the reaction catalyzed by either enzyme, indicating that the mechanism of oxygen activation is similar for both enzymes. The formation of the 4a-hydrate (II) from tetrahydropterin (I) is followed by the loss of water from II to yield the oxidized quinonoid species (III) as depicted in Scheme I.

Although the exact nature of the hydroxylating species itself is unknown, several possible structures have been proposed. They are (1) a 4a-peroxypterin; (2) an activated form of this species, such as a carbonyl oxide; (3) a 4a,8a-perepoxide; and (4) an activated metal–oxygen adduct or a 4a-peroxypterin–metal species (Hamilton, 1974; Bruice, 1982; Dmitrienko et al., 1977; Entsch et al., 1976; Benkovic & Lazarus, 1984; Benkovic et al., 1985a). In the first three cases above the function of the metal may be to facilitate the reaction of the pterin with oxygen and in the last case may have an additional role in the actual transfer of oxygen itself. Such roles would predict that the pterin and metal are in close proximity within the active site of the enzyme. In the following studies we have investigated the interaction of both [5-^{14}N]- and [5-^{15}N]-$DMPH_4$[2] and 5-deaza-6-methyltetrahydropterin with cupric PAH from *C. violaceum* using multifrequency EPR spectroscopy. The data indicate that a complex is formed between the cupric ion and the N-5 position in $DMPH_4$, supporting a functional relationship between the metal ion and the cofactor.

Experimental Procedures

Materials

6,7-Dimethyltetrahydropterin ($DMPH_4$) and 6-methyltetrahydropterin were synthesized by the methods of Mager et al. (1967) and Storm et al. (1971). 6,7-Dimethyltetrahydropterin containing ^{15}N in the 5-position (95%) was the gift of Dr. Wilfred L. F. Armarego, Department of Biochemistry, Australian National University, Canberra, A.C.T. 2601, Australia, and was prepared by methods previously described (Benkovic et al., 1985b). 6-Methyl-5-deazatetrahydropterin was prepared as described by Moad et al. (1979) and Stark and Breitmaier (1973). Reduced pterins were prepared by catalytic hydrogenation over Pt/C. Catalase was purchased from Boehringer-Mannheim as a suspension and diluted to 1 mg/mL in 20 mM Hepes, pH 7.4, before use. Bovine serum albumin, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), 2-(*N*-morpholino)ethanesulfonic acid (Mes), and L-phenylalanine were purchased from Sigma Chemical Co. Dithiothreitol (DTT) was purchased from either Aldrich Chemical Co. or Sigma. Doubly distilled water was used throughout these studies. All other reagents were of the highest grade commercially available.

Methods

UV spectra were recorded on a Cary 219 instrument. Fluorescence was measured on Perkin-Elmer MPF 44A and SLM Aminco 8000 instruments. Enzyme assays were performed on a Gilford 240 or 252 instrument. pH measurements were made with a Radiometer 22 instrument equipped with a Model PHA 630 Pa scale expander and a Radiometer GK-2302C electrode.

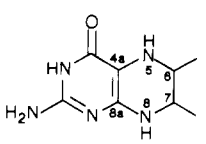
Enzyme Purification and Assays. C. violaceum PAH was purified by modification (Pember et al., 1986) of the procedure described by Nakata et al. (1979). The enzyme was assayed by monitoring the change in absorbance at 275 nm due to tyrosine formation (ϵ 1.7 mM^{-1} cm^{-1}; Miller et al., 1975). Protein was measured by the method of Lowry et al. (1951). The Bensadoun modification (Bensadoun, 1975) of the Lowry procedure was used when protein samples were in sulfonic acid buffers. The specific activity of PAH used in electron spin resonance measurements and fluorescence studies was between 10 and 12 units/mg of protein. The specific activity of enzyme used in inhibition studies was 6.8 units/mg of protein. An inhibition constant (K_I) was determined for 5-deaza-6-methyltetrahydropterin by double reciprocal plot analysis of enzyme activity in the absence and presence of inhibitor. The assay medium contained 5 μg of PAH, 50 μg of catalase, 6 mM dithiothreitol, 1 mM L-phenylalanine, and varying concentrations of $DMPH_4$ in 80 mM Hepes buffer, pH 7.4. When 5-deaza-6-methyltetrahydropterin was included, the concentration was 3.95 μM. The concentration of tetrahydropterin stock solutions was determined from UV absorption in 0.1 N HCl: $DMPH_4$, ϵ_{262} 16.0 mM^{-1} cm^{-1}; 6-MPH_4, ϵ_{262} 14.5 mM^{-1} cm^{-1}; 6-methyl-5-deazatetrahydropterin, ϵ_{265} 20 mM^{-1} cm^{-1}.

Fluorescence Measurements. The binding of 6-methyl-5-deazatetrahydropterin to *C. violaceum* PAH was measured by monitoring changes in enzyme fluorescence during titration with the cofactor analogue. Titrations were performed at 28 °C in 1-cm quartz cuvettes. Enzyme concentration was 1.5 μM, and the buffer solution was 0.1 M Hepes, pH 7.4. Excitation wavelength was at 300 nm, and emission wavelength was 332 nm. Although 6-methyl-5-deazatetrahydropterin has negligible absorbance at 300 nm, control experiments (using a tryptophan standard) were performed to ensure that the cofactor analogue did not contribute to fluorescence quenching. The enzyme fluorescence data were fit to the following equation: $Q = XQ_{ES} + (1 - X)Q_E$, where X is the mole fraction of complexed enzyme, Q is the observed signal, Q_{ES} is the fluorescence due to the binary enzyme complex, and Q_E is the signal due to uncomplexed enzyme. After X is determined, K_d is estimated from the relationship

$$X = [(K_d + [E_0] + [L]) - ((K_d + [E_0] + [L])^2 - 4[E_0] \times [L])^{1/2}]/2[E_0]$$

where E_0 is total enzyme, L the ligand concentration, and K_d the dissociation constant. Data were corrected for small effects

[2] Numbering system for the pyrazine ring of $DMPH_4$:



In comparison, 5-deaza-6-methyltetrahydropterin lacks the N-5 nitrogen and the 7-methyl group. 6-MPH_4 lacks only the 7-methyl group. Both $DMPH_4$ and 6-MPH_4 support phenylalanine hydroxylation by *C. violaceum* PAH; 5-deaza-6-methyltetrahydropterin does not.

due to dilution during the titrations. A linearized plot of the data was also used in the binding analysis as described by Gutfruend (1972).

ESR Spectroscopy. ESR measurements at The Pennsylvania State University were made on a Varian E-line spectrometer operating at X-band. A Model E-102 microwave bridge with a reference arm was used. In some experiments spectra were digitized and stored on magnetic disk with a Model S-100 microcomputer (Centre County Computer Consultants). At the National Biomedical ESR center (Medical College of Wisconsin) X-band spectra (ν 9.1 GHz) were obtained with a Varian E-109 Century Series X-band spectrometer. S-band spectra were obtained with a microwave bridge equipped with the loop-gap resonator cavity operating at 3.4 GHz (Froncisz & Hyde, 1982). Microwave frequencies were measured with an EiP Model 331 counter, and the magnetic field was calibrated with a MJ-110R Radiopan NMR magnetometer. Spectra were signal averaged by a Tractor Northern digital signal analyzer NS-570A and stored by a PDP 11/34 computer and a software program VIKING, written by Dr. C. Felix. PAH was buffered in 50 mM Mes containing 50 mM NaCl, pH 6.5. Enzyme concentration in these studies was $\simeq$800 μM. At least a 10-fold excess of pterin over enzyme was used in these experiments. Controls were also performed to ensure that further addition of pterin to enzyme solutions caused no further change in the spectra. All spectra in this work were recorded at liquid nitrogen temperatures ($\simeq$−150 °C).

Computer programs for simulation of frozen spectra of cupric complexes were obtained from Dr. J. Pilbrow, Monash University, Australia. Programs were simulated on a Masscomp computer, Model MCS-510A, which has 2-megabyte memory, a floating point unit, and UNIX and Fortran languages.

Results

X-Band ESR Spectroscopy of C. violaceum PAH in the Presence of $DMPH_4$ and 5-Deaza-6-methyltetrahydropterin. The cofactor analogue 5-deaza-6-methyltetrahydropterin does not support phenylalanine hydroxylation by *C. violaceum* PAH but is a good competitive inhibitor of the enzyme with an apparent K_I of about 1.0 μM (see Methods). To ascertain whether the binding of the cofactor analogue causes any perturbation in the cupric ion environment of the enzyme, the ESR spectrum of the enzyme was recorded in the presence of excess 5-deazapterin. Under these conditions there was no apparent change in the ESR parameters of the enzyme over those reported for the native enzyme: $g_\perp = 2.06$, $g_\parallel = 2.32$, and $A_\parallel = 157 \times 10^{-4}$ cm^{-1} (Pember et al., 1986, 1987). Since the active form of the enzyme contains cuprous ion, it seemed possible that the ESR data might be explained by an inability of the 5-deaza analogue to bind to the oxidized enzyme. This was investigated by monitoring cupric enzyme fluorescence during titration with the pterin analogue. As seen in Figure 1 there was a clear change in enzyme fluorescence that followed simple saturation behavior. A binding constant, K_d = 0.7 μM, was determined by computer fit of the data. This value is similar to the inhibition constant determined above. In addition, a linearized plot (Gutfreund, 1972) of the data (inset, Figure 1) indicated that there is a single pterin binding site on the enzyme (n = 1.03). From these data it is clear that 5-deaza-6-methylpterin binds to both oxidized and reduced enzymes but does not cause any appreciable change in the magnetic parameters associated with the cupric enzyme.

In contrast, when $DMPH_4$ was added to the cupric enzyme, the ESR parameters shifted: $g_\parallel$ changed from 2.32 to 2.27,

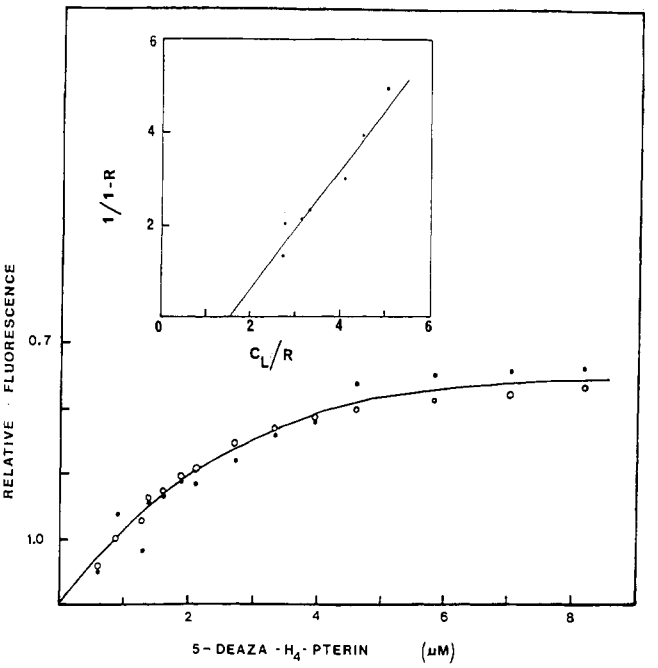


FIGURE 1: Fluorescence titration of *C. violaceum* PAH with 5-deaza-6-methyltetrahydropterin: (●) experimental data; (○) computed fit using the relationships described under Methods. The inset shows a linearized plot of the data using points in the middle of the titration curve. R is the fractional saturation, C_L is the deazapterin concentration, and the x intercept = nC_E, where n is the number of binding sites and C_E is the concentration of enzyme. See Methods for further details.

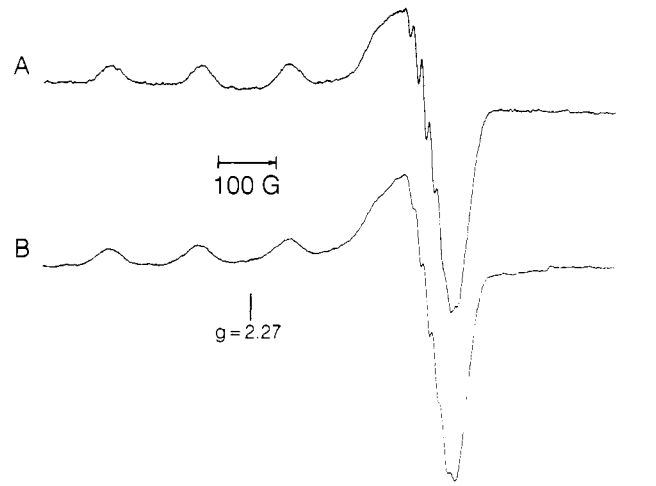


FIGURE 2: X-Band ESR first-derivative spectra for the cupric site in bacterial phenylalanine hydroxylase upon addition of a pterin cofactor in which the nitrogen in the 5-position is ^{14}N (A) or ^{15}N (B). The ESR instrument settings are as follows: temperature, about −150 °C; microwave power, 10 mW; microwave frequency, 9.084 GHz; time constant, 0.25 s; scan time, 4 min; modulation amplitude, 5 G; modulation frequency, 100 kHz.

and $A_\parallel$ from 157×10^{-4} cm^{-1} to 166×10^{-4} cm^{-1} (Figure 2A). In addition, superhyperfine transitions were resolved in the $g_\perp$ region. From the magnitude of the coupling ($\simeq$13 G) the transitions can be assigned to hyperfine splitting from nitrogen ligands (Wagner & Walker, 1983; Bereman & Kosman, 1977). Since the unpaired electron in the cupric ion is in the $d_{x^2-y^2}$ orbital, usually only nitrogen in the equatorial plane displays hyperfine splitting of the observed magnitude (Swartz et al., 1972; Walker et al., 1972). Similar changes were also observed for 6-MPH_4.

The ESR spectra recorded in the presence of 5-deaza-6-methyltetrahydropterin and $DMPH_4$ demonstrated that the

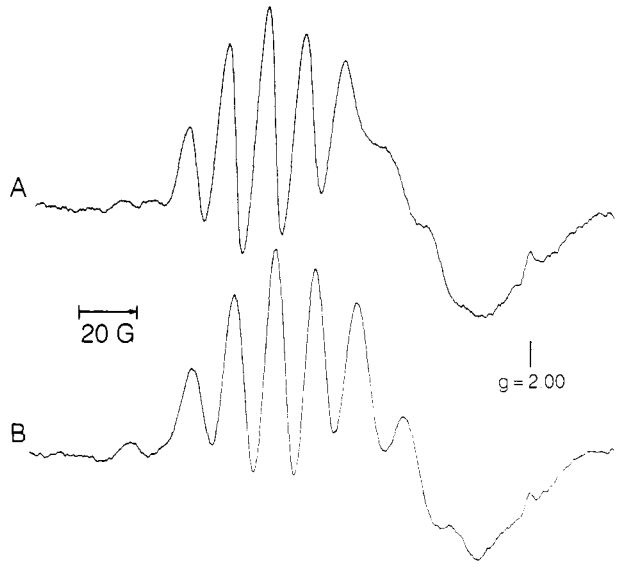


FIGURE 3: X-Band ESR second-derivative spectra for the $g_\perp$ region for the cupric site in bacterial phenylalanine hydroxylase upon addition of a pterin cofactor in which the nitrogen in the 5-position is ^{14}N (A) or ^{15}N (B). The ESR instrument settings are given in the legend for Figure 2 except the low-field modulation is 270 Hz (the high-field modulation frequency is unchanged from 100 kHz), the time constant is 0.3 s, and the scan time is 1 min. The spectra are the result of signal averaging of 21 (A) and 9 (B) scans, respectively. The signal on the high-field side of the scan is due to a free-radical contaminant, which is useful as an internal marker.

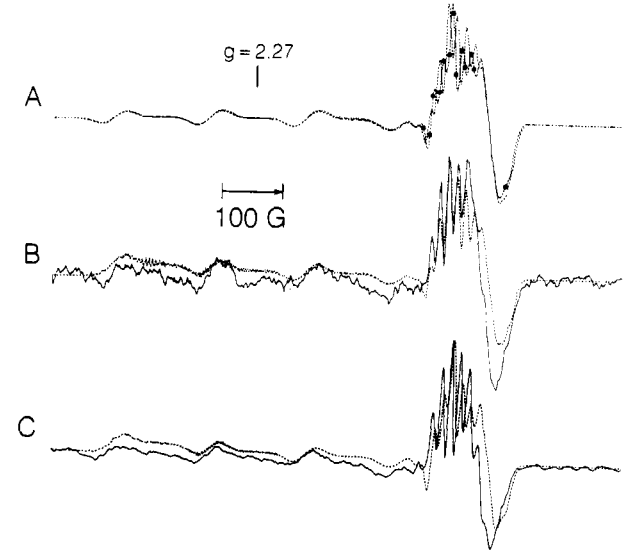


FIGURE 4: Simulated second-derivative X-band spectra for cupric ion bound to three equivalent ^{14}N donor atoms, Cu–3 ^{14}N (A, dashed line), or two ^{14}N donor atoms and one ^{15}N donor atom, Cu–2 ^{14}N + 1 ^{15}N (A, dashed line marked with filled circles). Simulated spectra (dashed line) compared to experimental spectrum for the cupric site in bacterial phenylalanine hydroxylase in the presence of the pterin cofactor in which the nitrogen in the 5-position is ^{14}N (B) or ^{15}N (C). The simulated spectra account for the presence of both isotopes of copper (71% ^{63}Cu and 29% ^{65}Cu). The experimental spectra are averaged from 4 scans (B) and 20 scans (C). The instrument settings are given in the legends for Figures 1 and 2. The ESR parameters for the simulated spectra are as follows: $A_\perp(^{14}N)$ = 12.98 G, $A_\parallel(^{14}N)$ = 10.44 G, $A_\perp(^{15}N)$ = 18.17 G (when appropriate), $A_\parallel(^{15}N)$ = 14.62 G (when appropriate); line width in perpendicular region 7.25 G; line width in parallel region 5.50 G.

nitrogen in the 5-position of the pyrazine ring is essential for perturbation of the copper environment. These data suggested the possibility that $DMPH_4$ might contribute to the total nitrogen superhyperfine pattern by coordination to the copper through the 5-position on the pyrazine ring, with the remainder of the nitrogen donor atoms coming from the protein itself.

To explore the possibility that $DMPH_4$ coordinates to the cupric center, ESR spectra were recorded in the presence of $DMPH_4$ labeled with ^{15}N in the 5-position. Because ^{15}N has a nuclear spin of $I = {}^1/_2$ and ^{14}N is $I = 1$, significant changes might be anticipated in the number and (or) position of resolved lines (Wertz & Bolton, 1972). Additionally, the magnitude of the coupling might also change due to the somewhat larger magnetic moment of ^{15}N (Wertz & Bolton, 1972; Simanek & Stroubek, 1972). As seen in Figure 2B the addition of [5-^{15}N]$DMPH_4$ caused apparent differences in line positions and line shapes in the $g_\perp$ region relative to [5-^{14}N]$DMPH_4$.

However, given the first-derivative display as shown in Figure 2, it is difficult to derive conclusive structural information about the copper binding site. Purified pterin-dependent phenylalanine hydroxylase contains both ^{65}Cu (31%) and ^{63}Cu (69%) isotopes. The positions of the ESR lines will not coincide because of the different magnetic moments for ^{63}Cu (2.2206) and ^{65}Cu (2.3790). Therefore, the ESR lines are not as well resolved as the lines from a site occupied by a single copper isotope. The $g_\perp$ region of the spectrum is additionally complicated because the hyperfine coupling constant for copper, $A_\perp^{Cu}$, is similar to the hyperfine coupling constant for nitrogen, $A_\perp^{N}$ ($\simeq$15 G). For example, if three equivalent nitrogen donor atoms are bound to cupric ion, the $g_\perp$ region consists of 28 hyperfine lines of which only about 7 are well resolved. Also, if the configuration is not perfectly square planar, i.e. $g_1 \neq g_2 \neq g_3$, then the $g_\perp$ region contains 56 lines. In addition to these lines, "overshoot" lines that occur from the particular angular dependence of the copper hyperfine lines often have appreciable intensity. Thus, it is difficult to quantitatively analyze the $g_\perp$ regions without computer simulation of the spectra.

The changes in line position in the $g_\perp$ region resulting from addition of [5-^{15}N]$DMPH_4$ are more evident from the second-derivative display (Figure 3). The center of both spectra consists of five intense well-resolved lines, but the positions of the lines are different for the copper site in PAH after addition of $DMPH_4$ in which the nitrogen in the 5-position is ^{14}N or ^{15}N (Figure 3). The low-field and high-field portions of the spectra also differ in line position and line shape. Computer simulations of spectra for copper complexes bound to three equivalent ^{14}N donor atoms, Cu–3 ^{14}N, or two ^{14}N donor atoms and one ^{15}N donor atom, Cu–2 ^{14}N + 1 ^{15}N confirm that the line positions should differ if these models are appropriate (Figure 4). Comparison of experimental and simulated model spectra suggest the ESR parameters for the simulated spectra are similar to, but not exactly equal to, the ESR parameters for spectra for PAH in the presence of [5-^{14}N]$DMPH_4$ and [5-^{15}N]$DMPH_4$ (Figure 4B,C).

S-Band ESR Spectroscopy of C. violaceum PAH in the Presence of [5-^{14}N]$DMPH_4$ and [5-^{15}N]$DMPH_4$. Resolution of copper powder ESR spectra can be improved at lower microwave frequencies, S-band, 2–4-GHz (Froncisz et al., 1979; Froncisz & Hyde, 1980; Froncisz & Aisen, 1982). Also improved resolution of nitrogen superhyperfine transitions have been reported at S-band in the carnosine–Cu(II) complex (Brown et al., 1980), bleomycin (Antholine et al., 1984), and 2-formylpyridine monothiosemicarbazone adducts with glutathione (Antholine & Taketa, 1984). Additionally, a more stringent test of the ESR parameters is to fit the experimental ESR spectra at two microwave frequencies, 3.4 GHz (S-band) as well as 9.1 GHz (X-band). S-Band spectra were therefore recorded for *C. violaceum* PAH in the presence of [5-^{14}N]-

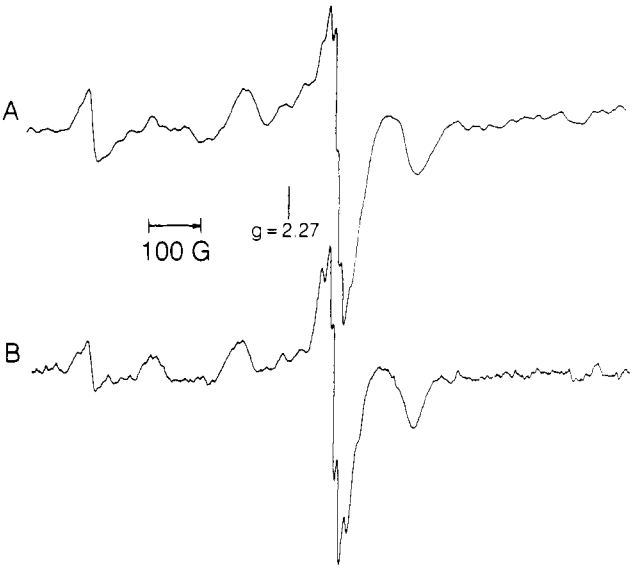


FIGURE 5: S-Band first-derivative spectra for the cupric site in bacterial phenylalanine hydroxylase in the presence of the pterin cofactor in which the nitrogen in the 5-position is ^{14}N (A) or ^{15}N (B). The spectra are averaged from 31 scans (A) and 4 scans (B). (The line on the low-field side is an iron signal.) The instrument settings are as follows: microwave frequency, 2.400 GHz; modulation amplitude, 5 G; time constant, 0.1 s; scan time, 0.5 min; microwave power, 2 dB; temperature, about −150 °C; modulation frequency, 100 kHz.

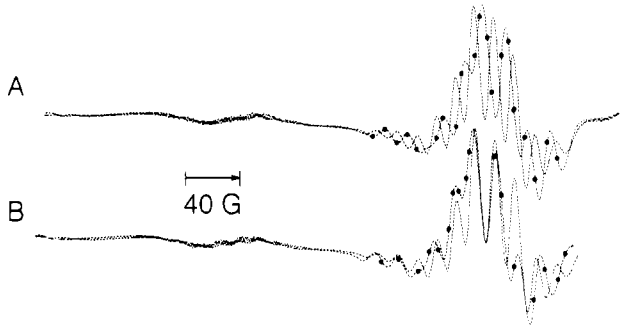


FIGURE 6: Simulated second-derivative S-band spectra for cupric ion bound to three equivalent ^{14}N donor atoms, Cu–3 ^{14}N (dashed lines), or two ^{14}N donor atoms and one ^{15}N donor atom, Cu–2 ^{14}N + 1 ^{15}N (dashed lines marked by solid circles). The simulated spectra account for the presence of both isotopes of copper (71% ^{63}Cu and 29% ^{65}Cu). In the lower spectra (B) the spectrum of Cu–2 ^{14}N + 1 ^{15}N has been shifted to match the most intense lines in the perpendicular region. The ESR parameters for the simulations are as follows: $g_\perp = 2.040$, $g_\parallel = 2.274$, $A_\perp^{Cu} = 7.20$ G, $A_\parallel^{Cu} = 158$ G, $A_\perp(^{14}N) = 13.65$ G, $A_\parallel(^{14}N) = 12.60$ G, $A_\perp(^{15}N) = 19.10$ G (when appropriate), $A_\parallel(^{15}N) = 17.64$ (when appropriate); line width in perpendicular regions 6.3 G; line width in parallel region 6.0 G; microwave frequency, 2.400.

$DMPH_4$ and [5-^{15}N]$DMPH_4$. As with the spectra at 9.1 GHz, the first-derivative S-band spectra for the cupric site in PAH in the presence of the isotopically labeled $DMPH_4$ cofactors differed slightly with respect to the number of resolved lines, the line shape, and line position of these lines in the region with the most intense lines (Figure 5A,B). As before, a second-derivative display was then used to facilitate the analysis of the data. First, spectra were simulated for Cu–3 ^{14}N and Cu–2 ^{14}N + 1 ^{15}N model complexes (Figure 6). These simulations confirm that the position and shape of the lines for the most intense set of lines should differ (Figure 6A). If the spectra are shifted such that the most intense well-resolved lines from Cu–3 ^{14}N and Cu–2 ^{14}N + 1 ^{15}N are superimposed (Figure 6B), one can more readily observe the relative changes in line positions and line shape for the rest of the lines (Figure 6B). Second, the simulated and experimental second-derivative spectra were compared. As seen in

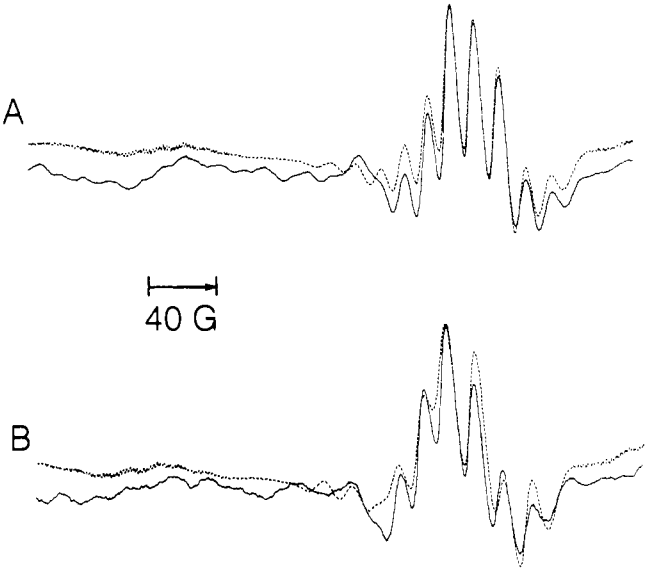


FIGURE 7: Experimental (solid lines) and simulated (dashed lines) S-band second-derivative ESR spectra for the cupric site in bacterial phenylalanine hydroxylase in the presence of the pterin cofactor in which the nitrogen in the 5-position is ^{14}N (A) or ^{15}N (B). ESR parameters for simulated spectra given in the legend for Figure 6.

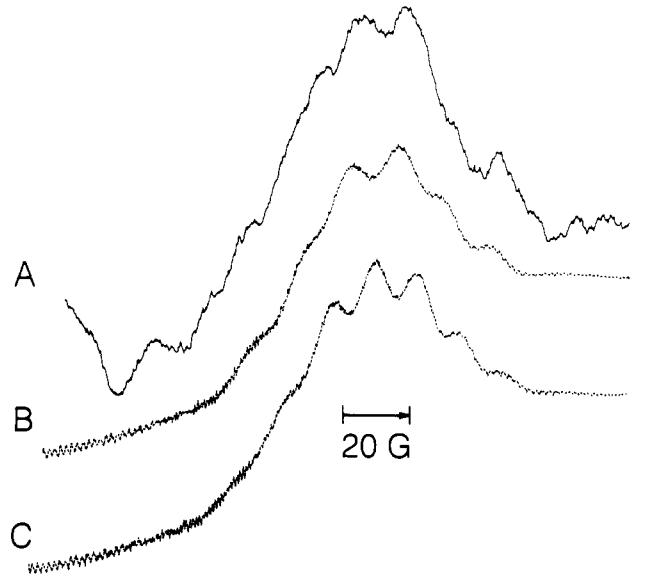


FIGURE 8: Experimental and simulated S-band first-derivative ESR spectra for the $M_I = -^1/_2$ line in the $g_\parallel$ region for the cupric site in bacterial phenylalanine hydroxylase in the presence of the pterin cofactor in which the nitrogen in the 5-position is ^{15}N (A) and simulated spectra for Cu–2 ^{14}N + 1 ^{15}N (B) and Cu–3 ^{14}N (C). ESR parameters for the simulations are given in the legend of Figure 6, except the line width for the parallel region was 4.0 G.

Figure 7A,B the spectral changes observed for the cupric site of PAH in the presence of cofactor in which either the nitrogen atom in the 5-position is ^{14}N or ^{15}N are well represented by the spectra for model cupric complexes in which three ^{14}N donor atoms or two ^{14}N and one ^{15}N donor atoms are bound to cupric ion.

Perhaps the best way to determine the number of nitrogen donor atoms bound to a single isotope of copper is to focus on the $M_I = -^1/_2$ line in the $g_\parallel$ region of the S-band spectrum (Froncisz & Hyde, 1980; Hyde et al., 1986). The difficulties associated with quantitating the $g_\perp$ region are essentially eliminated; however, when copper is present in natural abundance as in PAH, the pattern for the $M_I = -^1/_2$ line in the $g_\parallel$ region consists of lines from both copper isotopes (^{63}Cu and ^{65}Cu). We were able to resolve essential features of the

$M_I = -1/2$ line for PAH in the presence of ^{15}N-labeled $DMPH_4$ as shown in Figure 8A. Simulated spectra for complexes containing Cu–2 ^{14}N + 1 ^{15}N and Cu–3 ^{14}N are also given in Figure 8, parts B and C, respectively. Again, the experimental data are closely matched by the simulated spectra representing the Cu–2 ^{14}N + 1 ^{15}N complex in the number of resolved lines, line shape, and the magnitude of coupling. From the above studies we conclude that the nitrogen in the 5-position of the pterin cofactor serves as a donor ligand to the copper center in the enzyme.

Discussion

Multifrequency ESR data for PAH in the presence of its cofactor, $DMPH_4$, in which the nitrogen in the 5-position is either ^{14}N or ^{15}N strongly suggest that the nitrogen is an equatorial donor atom for the cupric site in PAH. The decrease in $g_\parallel$ and increase in $A_\parallel$ relative to the native enzyme suggests that an oxygen atom may be displaced for nitrogen upon $DMPH_4$ binding to the enzyme (Wagner & Walker, 1983; Formicka-Kozlowska et al., 1977). Isotopic substitution of ^{15}N for ^{14}N at the 5-position of the pterin cofactor was necessary to show that the nitrogen is coordinated to the copper in the equatorial plane. In the absence of the isotope effects the spectral changes could have been attributed to perhaps a reduction of g strain (Froncisz & Hyde, 1980) or an allosteric effect induced by the pyrazine ring.

A value of 2.27 for $g_\parallel$ and 166 × 10^{-4} cm^{-1} for $A_\parallel$ in the presence of $DMPH_4$ favors three nitrogens and one oxygen atom as donor atoms to the copper (Peisach & Blumberg, 1974). This is in agreement with the simulated data using Cu–3 ^{14}N or Cu–2 ^{14}N + 1 ^{15}N for PAH in the presence of [5-^{14}N]$DMPH_4$ or [5-^{15}N]$DMPH_4$, respectively. It should be emphasized that additional possibilities such as Cu–4 ^{14}N or Cu–3 ^{14}N + 1 ^{15}N cannot be completely eliminated at the present time. A more detailed analysis of the $M_I = -1/2$ line at S-band is needed to determine whether the hyperfine splitting pattern of PAH with $DMPH_4$ represents a 1-3-6-7-6-3-1 pattern for Cu–3 ^{14}N or a 1-4-10-16-19-16-10-4-1 pattern for Cu–4 ^{14}N, or perhaps a more complicated pattern. The relative ratios of the three central lines are almost identical: 86%, 100%, 86% for Cu–3 ^{14}N and 84%, 100%, 84% for Cu–4 ^{14}N. Differentiation between Cu–3 ^{14}N and Cu–4 ^{14}N is more evident from the relative intensities of the outer lines (Rakhit et al., 1985), but it is not yet clear whether the intensity and resolution of the outer resolved lines are sufficient to determine whether three or four equivalent nitrogen donor atoms are bound to the cupric ion site in PAH in the presence of $DMPH_4$. Similarily, the difference between the hyperfine patterns for 2 ^{14}N–1 ^{15}N, 1-2-1-3-2-2-3-1-2-1, and 3 ^{14}N–1 ^{15}N, 1-3-1-6-3-7-6-6-7-3-6-1-3-1, are difficult to analyze when many of the lines are not well resolved. Clearly, it would be helpful to know the number of nitrogen donor atoms in the absence of the cofactor, as well as to substitute a single copper isotope in the enzyme, but as yet, resolution of nitrogen fine structure in the native enzyme has not been possible and attempts at isotopic substitution in the enzyme have been unsuccessful.

The simulations of the data, particularly the simulations of the S-band spectra, model the experimental data well enough to evaluate the ESR parameters. A reasonable explanation for better simulations of the S-band spectra may be due to some rhombicity of the **g** tensor, i.e. $g_1 \neq g_2 \neq g_3$ instead of $g_\parallel$ and $g_\perp$. A difference in the in-plane g values, for example $\Delta g = 0.003$, would be difficult to observe with S-band (3 GHz), i.e. a separation of about 1.5 G; but evident with X-band (9 GHz), i.e., a separation of 4.5 G; and separated with Q-band (35 GHz), i.e. a separation of 18 G between g values. All the other parameters seem typical with the possible exception of the in-plane hyperfine coupling constant for copper, $A_\perp^{Cu} \simeq 7$ G. More typical values usually range from 10 to 20 G. Again S-band spectra may be less sensitive to this value because the intensity of the $M_I = -3/2$, $-1/2$, and $+3/2$ lines are less than the intensity of the $M_I = +1/2$ line. Additional simulations are necessary to determine the uniqueness of these parameters with respect to the **g** tensor, but certainly the model and experimental data are consistent with the equatorial binding of the nitrogen in the 5-position of pterin to the cupric ion.

Given these results it becomes important to determine whether the coordination of pterin is monodentate or bidentate; the most obvious structures give the nitrogen in the 5-position as an equatorial donor atom. Bidentate cupric complexes are most stable in a five-membered ring configuration. A tautomeric shift would give more negative character to the carbonyl oxygen and provide bidentate chelation. The following structures are suggested: one where pterin is a bidentate ligand and the other where pterin is primarily monodentate but stabilized by the axial coordination of the carbonyl oxygen. However, the metal center is likely not the only domain important for pterin binding to the enzyme since 5-deaza-6-methyltetrahydropterin also has high affinity for the protein.

An important question becomes whether the pyrazine ring–metal interaction is maintained upon reduction of the cupric ion to active cuprous form. A direct metal coordination to the 5-position of the pterin in mammalian PAH had previously been proposed during the formation of the active hydroxylating species (Benkovic & Lazarus, 1984). In this configuration the metal could serve as a Lewis acid or base to activate the 4a-position (more electrophilic or nucleophilic). Additionally, the incipient formation of a 4a-peroxy species could be polarized by close proximity to the metal ion, promoting effective oxygen transfer to the substrate. The necessity for a reduced transition metal also suggests the possibility of an initial coordination of molecular oxygen at the metal center. In any case, the current studies have demonstrated the close proximity of the pterin binding site and copper center in *C. violaceum* PAH and are supportive of a pterin–metal interaction in the mechanism of the enzyme.

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Positional Oxygen Isotope Exchange as a Probe for the Mechanism of Catalysis by *Escherichia coli* Succinyl Coenzyme A Synthetase†

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ABSTRACT: Succinyl-CoA synthetase of *Escherichia coli* has an $\alpha_2\beta_2$ subunit structure. The enzyme shows strict half-sites reactivity with respect to the phosphorylation of a histidine residue in the α subunit that represents a step in catalysis. Several lines of evidence indicate that this behavior may result from cooperative interactions between alternatingly functional active sites, so that subsequent steps in catalysis at one site may be promoted by phosphoryl transfer to the site on the neighboring half of the molecule. This study is directed toward learning more about the nature of these cooperative interactions. Here we have used positional isotope exchange (i.e., exchange of ^{18}O between the β,γ bridge and the β nonbridge position of ATP) as a test for transient bisphosphorylation. Succinyl-CoA synthetase was prepared in which one of the two active sites was thiophosphorylated; this species thus has one of its two active-site histidine residues occupied and unavailable for further reaction with ATP. Treatment of this monothiophosphorylated enzyme with [β,γ-^{18}O]ATP resulted in no significant scrambling of isotope into the nonbridge position, clearly indicating that the enzyme does not undergo even transient bisphosphorylation. We interpret the results in terms of a model of catalysis in which phosphoryl transfer to the second site occurs in concerted fashion with transfer from the first.

Succinyl-CoA synthetase catalyzes the substrate level phosphorylation step of the tricarboxylic acid cycle:

$$\text{succinyl-CoA} + \text{NDP} + P_i \rightleftarrows \text{succinate} + \text{NTP} + \text{CoASH} \quad (1)$$

The enzyme as isolated from *Escherichia coli*, the subject of this report, prefers adenine nucleotides as substrate and is of special interest because of the relationship of its quaternary structure to catalytic function. *E. coli* SCS[1] has been shown

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[1] Abbreviation: SCS, succinyl-CoA synthetase.

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